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Sex-specific reproductive trade-offs in the gregarious furoid macroalga *Ascophyllum nodosum*

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ABSTRACT

The existence of sex-specific reproductive trade-offs is well established in plants. They usually occur because females invest more resources into reproduction than males, and have to compensate by sacrificing growth or defence. Investigations into by-sex differences in resource allocation by seaweeds are comparatively scarce. A small number of authors report differences between the sexes in some red algae, but known by-sex differences in brown algae are largely confined to the reproductive structures. In this study, sex-specific reproductive trade-offs are investigated in the common temperate brown alga *Ascophyllum nodosum* from three distinct populations. Quantified investments into growth, defence, and reproduction of a large number of individuals ($n = 720$) selected across a full annual cycle are presented in parallel with feeding trials using a common gastropod herbivore and a comprehensive assessment of the biotic and abiotic stressors impacting on *A. nodosum* at three sites. These reveal that sex-specific reproductive trade-offs occur in two of the three populations, as females invest more into reproduction than males and are subsequently less chemically defended for the months post gamete release. Feeding trials confirm that this leaves females more vulnerable to grazing pressure during these months, although mortality and competitive ability appear unaffected in the field. Possible causes of the trade-offs made by females are discussed, and new avenues of investigation are identified which could reveal interesting parallels between seaweeds and higher plants.

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Introduction

In dioecious plants, males and females often diverge on resource allocation because their reproductive requirements differ. Females typically invest more energy into reproduction, and in fruit-bearing species, for example, they may require more water. These differences can be so pronounced that in many populations two sexes can occupy separate niches (Hesse & Pannell, 2011a). Differences between sexes most commonly manifest in sexual and reproductive traits (Delph, 2005), but disparities in size, growth rate, photosynthetic ability, defence against herbivores, and nutrient uptake have all been reported (Delph & Herlihy, 2012).

According to the resource allocation hypothesis, an individual's resource expenditure can be grouped into growth, maintenance (e.g. defence), and reproduction (Coley *et al.*, 1985), and these must be in balance. For example, in a stressful environment with high juvenile mortality, an individual may prioritize reproduction. However, growth and/or defences must be sacrificed to compensate, or if possible, the individual will increase the uptake of resources. Since males and females invest differently into reproduction, it is unsurprising that they often show differences in growth and susceptibility to herbivory (e.g.

Ågren, 1987; Delph, 1999; Obeso, 2002; Cornelissen & Stiling, 2005). These disparities can arise at different times of year, because both reproductive efforts and environmental stressors are often seasonal (Ågren, 1988; Cipollini *et al.*, 2013). Although much is known about sex-specific resource allocation in flowering plants (Barrett & Hough, 2012), comparatively little work has been undertaken in algae.

Production of secondary metabolites and susceptibility to herbivory have been shown to differ between sexes in seaweeds, for example during specific life-history stages in red algae (Vergés *et al.*, 2008; Payo *et al.*, 2011). Skewed sex-ratios have also been observed in certain populations, suggesting differential mortality (Engel *et al.*, 2001). However, in the brown alga *Fucus serratus*, the sexes display no differences in resource allocation apart from threshold size for reproduction, which is greater in females than males (Viejo *et al.*, 2011, but see Vernet & Harper, 1980). In the brown algal genus *Ectocarpus*, no differences are present between the sexes beyond the morphology of reproductive structures. Genetic analysis also reveals that external pressures act on males and females equally, although data have only been gathered from one population (Lipinska *et al.*, 2015). No study has yet directly quantified growth, defence,

reproductive effort and susceptibility to herbivory simultaneously in a dioecious alga across multiple populations for a full annual cycle. In this study, sex differences in resource allocation by *Ascophyllum nodosum* (L.) Le Jolis, a common, fully dioecious, temperate macroalga, are assessed.

Ascophyllum nodosum is perennial, long-lived (up to 30–40 years), and grows in dense, monospecific stands that result in intense intraspecific competition for space and light (Åberg, 1992). As such, the vegetative tissues of *A. nodosum* have significantly higher 'fitness values' (i.e. the contribution to the success of the individual) than its reproductive tissues (see Pavia *et al.*, 2002), which it produces in vast abundances. These often equal the mass of its vegetative tissues during the reproductive season (Åberg, 1996; Pavia *et al.*, 2002). Therefore, it is predicted that differences between sexes are unlikely to manifest in size, since the competitive and reproductive disadvantage experienced by a smaller sex would have considerable negative ecological consequences (e.g. Bessa-Gomes *et al.*, 2004; Hesse & Pannell, 2011b). However, given that female *A. nodosum* probably invest more into reproduction than males (Vernet & Harper, 1980), it is expected that differences in resource allocation will be detectable in the reproductive tissues. If present, this higher expenditure must then be balanced by reduction in either growth or defence.

Being a sheltered-shore species, *A. nodosum* is negatively impacted by strong wave action, and success of its germlings (zygotes) is highly dependent on them settling within the established stand (Vadas *et al.*, 1990), where even then grazing by gastropods can result in extremely high post-settlement mortality (Cervin & Åberg, 1997; Dudgeon & Petraitis, 2005). Defensive investment is strongly dependent on levels of herbivory, and the production of defensive compounds has negative impacts on growth (Pavia *et al.*, 2002; Toth *et al.*, 2007). Herbivory also causes frond breakage, and therefore has substantial negative impacts on individual survivability and reproductive output (Cousens, 1985; Åberg, 1996; Pavia & Toth, 2000; Davies *et al.*, 2007). However, it is expected that females must be lesser-defended and subsequently more susceptible to herbivory than males, in order to balance the costs of reproduction. Therefore, mortality may differ between the sexes, because of the reproductive handicap experienced by females.

In plants, phenotypic plasticity in response to environmental conditions may facilitate sexual dimorphism and other sex-specific traits in some species (Barrett & Hough, 2012). However, because of the physiological simplicity of *A. nodosum*, lack of internal transport systems, and the absence of any known internal feedback mechanism associated with reproductive success (given that fertilization is external), it is unlikely that plasticity will explain any

differences between sexes that are observed in this alga. By monitoring investment into the three allocations of growth, defence and reproduction for a full annual cycle in three populations, the following hypotheses were tested. (1) Females will allocate more resources to reproduction. (2) Males and females will be of similar size. (3) Females will invest less into defence, leaving them more susceptible to herbivory. (4) Phenotypic plasticity will not explain sex-specific responses. Testing these hypotheses advances our knowledge of sex-specific resource allocations, within populations that experience intense intraspecific competition.

Materials and methods

Study organism

Ascophyllum nodosum is a large (1–3 m long), common, mid-intertidal macroalga found on temperate sheltered rocky shore habitats. Vegetative lateral shoots are seldom broader than 10 mm or thicker than 3 mm and so frequently break, particularly under high degrees of wave exposure and herbivory, but individuals still survive and continue to grow provided the holdfast remains attached (Cousens, 1985; Toth *et al.*, 2007). Receptacles (reproductive structures) are produced in pairs from pits on the vegetative shoots, and although individual size is directly related to fecundity, production of receptacles does not directly influence somatic growth because they and their supporting structures are separate from the meristems. Receptacle size positively correlates with individual size, but the number of gamete-producing structures within the receptacles (conceptacles) does not usually differ between individuals (Åberg, 1996). Timing of receptacle production differs depending on latitude but typically begins in mid-summer, and receptacles are shed after releasing the externally fertilized gametes during a 2-week period at the end of the following spring (Cousens, 1985; Pavia *et al.*, 2002; Dudgeon & Petraitis, 2005). Recruitment is negligible beyond the boundaries of stands and migration of zygotes is extremely unlikely to occur between them (Dudgeon *et al.*, 2001). This probably results in limited gene flow between even local (< 10 km) populations (Olsen *et al.*, 2010).

Like other members of the Fucales, *A. nodosum* produces phlorotannins as defensive compounds in response to herbivory and UV exposure (Pavia *et al.*, 1999). These compounds are inducible by direct herbivory from slow-moving gastropods, and by water-borne cues from nearby conspecifics. However, they are not induced by more motile crustacean herbivores, or by artificial clipping (i.e. simulated herbivory) (Pavia & Toth, 2000). The production of phlorotannins is carried out at the expense of growth,

and is therefore undertaken only as required (Toth & Pavia, 2007). However, production of phlorotannins is not known to be dependent on the abundance of growth-limiting micronutrients such as nitrogen (Toth *et al.*, 2007). In this study, *A. nodosum* individuals were differentiated as per Åberg (1989), and their sex determined by the colour of the conceptacles (Dudgeon *et al.*, 2001), males being orange and females being green (validated by light-microscopy for the presence of antheridia and oogonia, and found to be valid from December to April).

Site selection

Sampling was conducted on the Isle of Anglesey, North Wales, an area central in the latitudinal range of *A. nodosum*. The region has an average sea surface temperature of ~16°C, and a tidal range of ~6 m. The island is small enough that differences in UV-radiation, temperature and salinity are negligible around its coast. However, wave-exposure and herbivore abundances differ between rocky shores on the island, and because resource allocations are known to differ between *A. nodosum* stands depending on both biotic and abiotic conditions (Cousens, 1985, 1986; Strömberg, 1986; Araújo *et al.*, 2015) the experiment was repeated at three distinct locations. The three sites selected were: (1) Ynys Faelog near Menai Bridge (53.226793°N, 4.157054°W) (hereafter Menai); (2) Cemlyn Bay near Cemaes (53.414750°N, 4.511564°W) (hereafter Cemlyn); (3) Bull Bay, near Amlwch (53.422543°N, 4.368959°W) (hereafter Bull).

The wave-stress of each site was estimated from fetch distance of open water at 10° compass intervals emanating from the sites (Supplementary fig. 1, see Davies & Johnson, 2006). Longer distances lead to the generation of greater wave height by winds, and therefore have a significant impact on the species composition of a site (Lindegarth & Gamfeldt, 2005). A survey then established the magnitude of biotic and abiotic stressors at each site using several metrics and to determine whether by-sex differences existed in mortality (see Engel *et al.*, 2001) within the *A. nodosum* populations. (1) The sex ratio between male and female *A. nodosum* at each site was determined by a random, non-destructive survey of 100 individuals at each site in April. (2) The modelled exposure estimates were verified by placing gypsum (CaSO₄) clod cards (Thompson & Glenn, 1994) in the general area of the *A. nodosum* population on each of the identified shores, taking care to ensure that the clods were not touched by the surrounding algal stands ($n = 14$ at each site). Clod dissolution correlates with mean flow conditions, and is an established method that has proven to demonstrate differences in wave exposure levels between sites (Jonsson *et al.*, 2006). Clods were moulded in plastic trays from a

mixture of 1.5 kg gypsum to 1 l water. The clods, 4.2 × 3.4 × 3.5 cm, were dried at 45°C for 48 hours, before being sanded to a consistent dry mass of 27 g (± 0.01 g). Deployment at each site was for 12 tidal cycles during August. After recovery, clods were dried again for 48 hours at 45°C and proportional dissolution calculated from the change in mass. (3) The background sediment composition of each site was established by taking three replicate sediment samples (~500 g) from each site within the boundaries of the *A. nodosum* stand. Sediment samples were washed with fresh water and dried for 48 hours at 110°C. After manually breaking up adhered clumps, samples were mechanically sieved into ϕ components (Phi, a logarithmic scale of particle size) using sieves with mesh sizes at quarter ϕ intervals, and resultant fractions weighed to two decimal places (see Blott & Pye, 2001). Sediments finer than 63 μ m were analysed using a Malvern Mastersizer laser particle-sizer. All sediment analysis was conducted using the Gradistat software (version 4, Blott & Pye, 2001). More exposed sites generally contain larger sediments (i.e. sand, gravel) compared with sheltered sites (i.e. mud, silt). (4) The intensity of gastropod herbivory was estimated from the mean abundance of snails known to consume *A. nodosum* (*Littorina littorea*, *Littorina fabalis*, *Littorina obtusata* and *Patella vulgata*) on each shore, estimated from ten 0.25 m² quadrats thrown haphazardly within the *A. nodosum* population (Toth *et al.*, 2007). This was done only once in the month before the annual survey began, as herbivore composition and abundance is unlikely to change significantly throughout the year (Toth & Pavia, 2007). *Littorina fabalis* was included in surveys because although it has been previously shown to consume algal films (Viejo & Åberg, 2003), it does also consume macroalgae including *Sargassum muticum*, *F. serratus*, and *A. nodosum* (M. Kurr, unpublished observations).

Sampling and analysis protocols

For one year, at the start of each month, 10 males and 10 females of *A. nodosum* were selected at random (using random steps) from each site (60 per month, 240 per site, 720 different individuals in total). Several protocols were undertaken: (1) Algal dry mass (DM) was estimated *in situ* for each alga by measuring the length (l) and circumference (c) of each individual to the nearest half centimetre, and applying the formula $DM = 0.057 (lc^2)^{0.7900}$ for winter (when receptacles are full), and $DM = 0.034 (lc^2)^{0.8605}$ for summer (when receptacles are small) (Åberg, 1990). (2) Five to six undamaged meristematic apices were torn off by hand to avoid inducing phlorotannin production by using other means (Pavia *et al.*, 1997). (3) From December to April, the receptacles (reproductive

bodies) of all reproductively active adults were sufficiently developed that sexes could be differentiated. A sub-sample of ~100 receptacles was sampled from the same fronds as the sampled meristems to avoid bias from potential intra-individual differences (Cousens, 1985) (60 individuals per month, 100 per site, 300 in total). Receptacles are shed after April and to allow the sampling to continue beyond this period, 100 males and 100 females were identified and tagged at each site in this month. No reproductive material was collected between May and November to avoid bias for those that shed receptacles later, or began developing them earlier than most of the population.

Investment into reproduction

Energy content of reproductive algal tissues was estimated by ascertaining Ash-Free Dry Mass (AFDM) per unit DM as a proxy for the percentage of organic compounds in the tissues by mass (Littler & Littler, 1985). Samples were cleared of epiphytes, washed thoroughly with distilled water, freeze-dried to constant mass, homogenized, and a known quantity (~1000 mg) was weighed to the nearest milligram. This was ashed-off at 450°C for 3.5 hours, and reweighed (Holme & McIntyre, 1984). In algae, this technique provides almost identical results to calorimetry (Lamare & Wing, 2001).

Ascophyllum nodosum produces a large quantity of receptacles, and enumerating them for all individuals across the observed period was not possible. However, because the abundance and size of receptacles differs between individual *A. nodosum* (Åberg, 1996), an estimate of receptacle abundance and mass for male and female individuals at each site was calculated. Fifteen male and 15 female *A. nodosum* individuals were collected from each of the three sites in March ($n = 90$). March was selected for collection, as at this time the receptacles were almost fully ripe, but none had discharged. Estimated DM of the individuals was ascertained *in situ* as above and samples were bagged whole, taking care not to damage the fronds or cause loss of receptacles. The receptacles were then systematically removed by hand, and counted. A random subsample of exactly 100 receptacles from each individual was taken, freeze-dried to constant mass, and weighed to the nearest milligram. The DM of the subsample was extrapolated to the total receptacle count, to estimate the total DM of receptacles produced by each individual. An estimation of the relative reproductive investment (RRI) for each individual was then calculated by the formula:

$$RRI = \left(\frac{ab}{c} \right) 100$$

where a is the estimated dry mass of all receptacles produced by the alga, b is the mean AFDM per unit DM of receptacles from that alga's respective site and

sex (ascertained from the receptacles of individuals collected for the annual survey, $n = 300$ individuals, ~100 receptacles each), and c is that individual alga's total estimated DM.

This proportional index of reproductive effort estimates the energy (AFDM per unit DM) allocated per unit DM of alga, by each of the 90 individuals into its receptacles, averaged across the 5 months when receptacles were visible (December–April). It proves useful here because it does not depend on individual size (Regression analysis $n = 90$, $RRI = 10.84 - 0.00334 DM$, $R^2 < 0.001$, $P = 0.618$), unlike other estimations of reproductive effort (e.g. Åberg, 1996).

Investment into defence

The phlorotannin abundance of both meristematic apices and receptacles (when collected) for each individual (10 males and 10 females, per site, per month) was determined using a modified version of the Folin–Ciocalteu method (Van Alstyne, 1995). Initially, tissues were washed thoroughly with distilled water and cleared of epiphytes. Samples were then freeze-dried to constant mass, ground until homogenous, and 0.2 g subsampled for phlorotannin extraction (60% aqueous acetone used as a solvent). Phlorotannins were extracted under constant agitation in the dark for 1 hour. The algal pulp was separated from the supernatant by centrifugation (5300 rpm for 10 min) and the acetone removed using *in vacuo* cold distillation (80 kPa, 38°C) until only the 40% aqueous fraction remained (typically ~2 hours). This was then diluted, filtered to remove precipitated lipophilic compounds (Pavia & Toth, 2000), and a 1 ml subsample was diluted in 11 ml of water. To this extract, 1 ml of Folin–Ciocalteu's phenol reagent (Sigma-Aldrich F9252) was added, followed immediately by 12 ml of 1M aqueous sodium carbonate decahydrate solution (Sigma-Aldrich 71360) to act as a buffer. The resultant solution was incubated in the dark for 30 min and analysed by spectrophotometry at 760 nm, using phloroglucinol (1,3,5-trihydroxybenzene, Sigma-Aldrich P3502) as a standard (Van Alstyne, 1995). Each month, 12 randomly selected samples (six male and six female) were analysed in triplicate to quality control the phlorotannin protocol. Replicates were never more than 0.1 standard deviations from their respective means. Defensive investment was expressed as the percentage of algal dry mass composed of phlorotannins. Relative defensive investment of receptacles was estimated by expressing receptacle phlorotannin abundance as a fraction of vegetative phlorotannin abundance.

Role of algal sex on herbivore preference

Littorina obtusata L. is a common consumer of *A. nodosum* found throughout Western Europe, and was

used as a model herbivore in laboratory-based feeding trials. This species shows a feeding preference towards algal tissues containing lower levels of phlorotannins, making it an indicator for biologically relevant differences in defensive investment (Pavia & Toth, 2000; Pavia *et al.*, 2002).

Feeding trials were undertaken across a 5-month period between May and October (hereafter 'summer trials'), and a 3-month period between January and March (hereafter 'winter trials'). Due to space limitations, trials were undertaken as a series of blocks, which were separated by between one and three weeks throughout the 'summer' and 'winter' periods. Each block was of the same design: one male and one female *A. nodosum* was selected from each of the three sites on one day ($n = 6$) and DM estimated as above. Samples were maintained in ambient flow-through seawater overnight before each trial began. From each individual, nine 500 mg blotted wet mass (± 50 mg) pieces of meristematic apical tissue were sampled, cleared of epiphytes and weighed to the nearest milligram (Pavia *et al.*, 2002). All manipulation was done by tearing the tissues by hand in order to simulate physical damage and therefore limit artificial phlorotannin induction (Pavia *et al.*, 1997). Most shoots contained an airbladder, and this was punctured to make all pieces negatively buoyant, thereby avoiding encounter rate bias. Of the nine pieces, three were used as no-grazer controls to correct for autogenic changes in mass, three were used in no-choice feeding trials, and three were used in choice feeding trials. An additional three to four apical shoots were also collected for phlorotannin analysis.

All *L. obtusata* were collected seven days prior to experimentation from each of the study sites, and starved to ensure an even level of hunger. Each *L. obtusata* was used in one trial only with *A. nodosum* from its site of origin, and no *L. obtusata* collected died during starvation or experimentation ($n = 1728$). All trials were undertaken for 10 days in outdoor aquaria, supplied with ambient flow-through seawater ($\sim 16^\circ\text{C}$ summer, $\sim 9^\circ\text{C}$ winter, 34 psu, ~ 30 seconds flushing time). Each individual aquarium (270 ml volume) was positioned partially submerged in a water bath to limit fluctuations in temperature, which was itself under a roof to prevent rainwater contaminating the system and to reduce the impact of air temperature on the aquaria. No individual aquaria were connected to any other to ensure independence, and to remove any potential for chemical signalling between treatments. Photosynthetically relevant levels of incident radiation (400–700 nm) under the roof were $\sim 25\%$ ambient (measured with an MDS-MkV/L Adventec photometer on a sunny day at noon, in July).

For no-choice feeding trials, one piece of algae was presented to three *L. obtusata*. For choice feeding-

trials, each piece was paired with one from the opposite sex from that respective shore, and presented to six *L. obtusata* collected from within that *A. nodosum* stand. Coloured string was tied around each piece of algae in the choice trials to discern male from female. At the end of the trial all pieces were then blotted dry and reweighed to the nearest milligram. Eight blocks of trials were undertaken in summer ($n = 48$), and eight blocks of trials were undertaken in winter ($n = 48$). Different *A. nodosum* individuals were selected for each block giving a total of 96 individuals used (48 in summer and 48 in winter). There was no effect of block on the consumption of tissue in summer (one-way ANOVA: $F_{7,137} = 1.94$, $SS = 252347$, $P = 0.068$) or winter trials (one-way ANOVA: $F_{7,133} = 1.88$, $SS = 235767$, $P = 0.077$).

Statistical analyses

Differences in clod dissolutions and grazer densities between sites were assessed with one-way Analysis of Variance (ANOVA), and means compared with Tukey's HSD *post hoc* procedures. Sex-ratios were tested for significant deviation from 1:1 using chi-square analyses. The effects of sex and time on dry mass, phlorotannin concentration, and energy content in *A. nodosum* tissues from each site were assessed using two-way ANOVA, with both factors fixed and orthogonal. Because *A. nodosum* displays a distinct seasonality in its production of phlorotannins (Ragan & Jensen, 1978), separate two-way ANOVAs were undertaken for summer months (when phlorotannin levels are rising) and winter months (when phlorotannin levels are falling), in line with the timings of the feeding trials. Means were compared using Tukey's HSD *post hoc* procedures. Differences in the consumption of different *A. nodosum* sexes by *L. obtusata* were assessed using one-way ANOVA. Due to the length of time over which feeding trials were undertaken (5 months for summer trials, 3 months for winter trials), phlorotannin abundances (which vary with season) were different between individuals used in the first and those used in the last blocks. To compensate, differences in phlorotannin abundance between tissues used in feeding trials were assessed using paired *t*-tests. Levene's test was used to ensure all data conformed to the assumptions of homogeneity of variance. In the event of non-conformity, data were either transformed or Welch's ANOVA was used and means compared with Game's Howell *post hoc* tests (Domingues *et al.*, 2007).

Finally, the Relative Distance Plasticity Index (RDPI) was used to estimate the amount of plasticity in the measured responses of *A. nodosum* following Valladares *et al.* (2006). This index ranges between 0 (no plasticity) and 1 (maximum plasticity) and can be used to evaluate trait plasticity across environmental

conditions (e.g. sites, as in the present study). Pairwise RDPI was calculated as the value of the difference in trait values of all male and female individuals within the sampled populations, (1) Menai to Cemlyn, (2) Cemlyn to Bull and (3) Menai to Bull. These pairwise values can be treated as replicate measures of distance, and were assessed using one-way ANOVA after arcsine transformation, with sex as a fixed factor.

Results

Between-site differences

All biotic and abiotic metrics demonstrated significantly different conditions in wave energy and grazing intensity from the sampled sites. Fetch distances (Supplementary fig. 1), grain size analysis (Supplementary fig. 2), and clod dissolution (one-way ANOVA, $F_{2,39} = 300.88$, $SS = 3.368$, $P < 0.001$) all indicated that wave exposure increased incrementally between sites in the order Menai < Cemlyn < Bull. Total herbivore density also followed this trend (one-way ANOVA, $F_{2,27} = 90.97$, $SS = 80021$, $P < 0.001$), as did the densities of most herbivore species. Only *Patella vulgata* were more abundant at Cemlyn than Bull, although these were patchily distributed and frequently absent from quadrats at the medium-stress site ($\bar{x} = 18 \text{ m}^{-2}$, $SD = 19.24$). There was no significant difference in the sex ratio of *A. nodosum* at any site (Menai: $n = 100$, $\chi^2 = 0.64$, $P = 0.424$, Cemlyn: $n = 100$, $\chi^2 = 1.44$, $P = 0.230$, Bull: $n = 100$, $\chi^2 = 2.56$, $P = 0.110$) suggesting a lack of sex-specific mortality.

Investment into growth and reproduction

Populations of *A. nodosum* exhibited significant differences in individual dry masses (Welch's ANOVA $F_{2,431.107} = 21.13$, $P < 0.001$, Game's-Howell *post hoc*, Menai > Cemlyn > Bull). However, males and females within each population had similar dry masses at all three sites throughout the year (Table 1). Investment into receptacles during the spring meant individuals had larger estimated dry masses at this time of the year, and although there were subtle differences in the timing this pattern was broadly similar across the three sites (Table 1).

By-sex differences were present in the energy-content of reproductive tissue. There was less energy in male reproductive tissues ($\bar{x} = 49.60\%$, $S.E. = 0.35$) than in female reproductive tissues ($\bar{x} = 54.86\%$, $S.E. = 0.56$) from all three sites. This pattern was consistent over time at Menai (Table 2a), and variable at Cemlyn (Table 2b) and Bull (Table 2c). Female receptacles at Bull contained significantly more energy in December and January, although at Cemlyn there

Table 1. Dry masses of *A. nodosum* males and females collected from (a) Menai, (b) Cemlyn and (c) Bull, across a 12-month period from December to November analysed using two-way ANOVA. * indicates results from Tukey's HSD *post hoc* analyses for significant effects, reported largest first (groups that do not share a number are significantly different).

(a)				
	DF	MS	F	P
Sex	1	60525	1.55	= 0.214
Month*	11	427047	10.95	< 0.05
Sex × Month	11	7705	0.20	= 0.998
Residual	216	38994		

*Month: (1) Dec, Jan; (1, 2) Mar; (2, 3) Apr, Feb; (3) Nov, Jun, Oct, Jul, Sep, May, Aug.

(b)				
	DF	MS	F	P
Sex	1	70086	3.55	= 0.061
Month*	11	65618	3.32	< 0.05
Sex × Month	11	15787	0.80	= 0.640
Residual	216	19742		

*Month: (1) Feb, Mar; (1, 2) Jan, Dec, Apr, Oct, Sep, Nov, Jun, Jul, May; (2) Aug.

(c)				
	DF	MS	F	P
Sex	1	29513	3.52	= 0.062
Month*	11	25062	2.99	< 0.05
Sex × Month	11	6142	0.73	= 0.708
Residual	216	8395		

*Month: (1) Mar; (1, 2) Jan; (1, 2, 3) Nov, Apr, Dec, Sep, Feb, Oct, May, Aug; (2, 3) Jul; (3) Jun.

were no significant differences in any one month (Table 2b).

Similar RRI's were estimated from male and female *A. nodosum* at Menai (one-way ANOVA, $F_{1,29} = 0.22$, $SS = 29.60$, $P = 0.644$) and Cemlyn (one-way ANOVA, $F_{1,29} = 3.31$, $SS = 35.07$, $P = 0.080$). However, at Bull female RRI was higher than that of males (one-way ANOVA, $F_{1,29} = 19.4$, $SS = 272.10$, $P < 0.001$; Fig. 1).

Investment into defence

Throughout the year, *A. nodosum* from all three sites demonstrated a sinusoidal pattern of phlorotannin production in meristematic tissue, being lowest at the end of spring and highest in winter (Fig. 2). When significant differences in phlorotannin abundance were observed between sexes, males were better defended than females. However, these differences existed only during the summer once receptacles had been shed (April–September), and were absent from Menai (Fig. 2a, Table 3a), infrequent at Cemlyn (Fig. 2b; Table 3b), and consistent across the whole of summer at Bull (Fig. 2c,

Table 2. Ash-free dry mass per unit dry mass in the reproductive tissues of *A. nodosum* from (a) Menai, (b) Cemlyn, (c) Bull, collected across a 5-month period from December to April analysed using two-way ANOVA. * indicates results from Tukey's HSD *post hoc* analyses for significant effects, reported largest first (groups that do not share a number are significantly different).

(a)				
	DF	MS	F	P
Sex*	1	1295	53.62	< 0.05
Month**	4	97	4.00	< 0.05
Sex × Month	4	56	2.30	= 0.065
Residual	90	24		

*Sex: (1) Female; (2) Male.

**Month: (1) Dec; (1, 2) Jan, Apr; (2) Feb, Mar.

(b)				
	DF	MS	F	P
Sex	1	274	14.49	< 0.05
Month	4	376	19.84	< 0.05
Sex × Month*	4	61	3.22	< 0.05
Residual	90	19		

*Sex × Month: (1) F Dec; (1, 2) F Jan; (1, 2, 3) M Dec; (2, 3, 4) F Feb; (2, 3, 4, 5) M Jan; (3, 4, 5) F Apr; (4, 5) M Mar, M Feb, M Apr; (5) F Mar.

(c)				
	DF	MS	F	P
Sex	1	690	33.48	< 0.05
Month	4	21	0.99	= 0.415
Sex × Month*	4	70	3.37	< 0.05
Residual	90	19		

*Sex × Month: (1) F Dec; (1, 2) F Jan; (1, 2, 3) F Feb, F Mar, F Apr, M Mar; (2, 3) M Apr; (3) M Jan, M Feb, M Dec.

Table 3c). No by-sex difference in phlorotannin abundance existed at any site over the winter (October–March) (Fig. 2, Table 4).

There was no significant difference between phlorotannin abundance in male and female reproductive tissues in *A. nodosum* from any site across the reproductive period (two-way ANOVA: Menai Sex × Month $F_{4,99} = 1.32$, MS = 0.10, $P = 0.268$, Cemlyn Sex × Month $F_{4,99} = 1.69$, MS = 0.22, $P = 0.160$, Bull Sex × Month $F_{4,99} = 1.40$, MS = 0.14, $P = 0.241$). However, because of the significant decrease in vegetative phlorotannin levels in females from Cemlyn and Bull during April, when normalized for levels of vegetative defence, female reproductive tissues were relatively better defended than male reproductive tissues from those two sites (Table 5).

Influence of sex on herbivore preference

Menai *L. obtusata* exhibited no preference for feeding upon either male or female tissue in either summer (one-way ANOVA: $F_{3,81} = 0.1$, SS = 7249, $P = 0.959$, Fig. 3a) or winter (one-way ANOVA: $F_{3,86} = 1.01$, SS = 29 620, $P = 0.392$, Fig. 3b) choice and no-choice feeding trials. In addition, there was no significant difference between summer (male $\bar{x} = 2.65\%$ DW, S.E. =

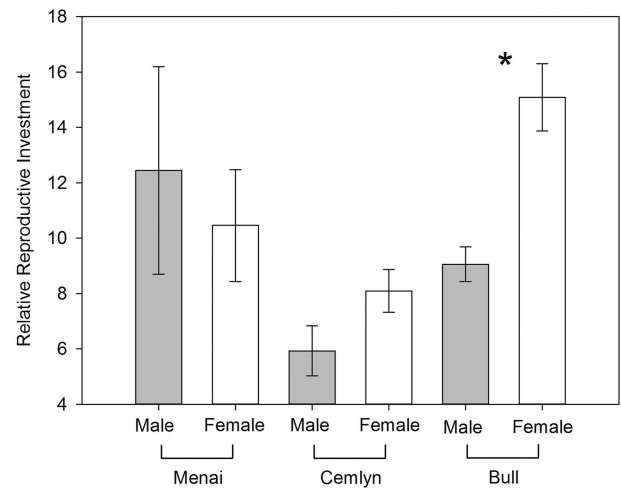


Fig. 1. Estimated relative reproductive investment (RRI) of male (grey bars) and female (white bars) *A. nodosum* collected from the three sites in March; calculated as ash-free dry mass of reproductive material per unit dry mass of total algal mass. Each bar represents 15 individuals ($n = 90$), error bars show ± 1 S.E. * indicates where differences between males and females were significant, based on Tukey's HSD *post hoc* tests ($P < 0.05$).

0.18; female $\bar{x} = 2.58\%$ DW, S.E. = 0.17; Paired *t*-test, $T_7 = 0.59$, $P = 0.574$) or winter (male $\bar{x} = 2.75\%$ DW, S.E. = 0.1; female $\bar{x} = 2.62\%$ DW, S.E. = 0.06; Paired *t*-test, $T_7 = 0.82$, $P = 0.438$) phlorotannin abundance in *A. nodosum* used during these trials.

When presented with the option to feed on either male or female *A. nodosum* in summer choice trials, Cemlyn *L. obtusata* preferred female tissue (one-way ANOVA: $F_{1,47} = 6.86$, SS = 114013, $P < 0.05$, Fig. 3c). However, when presented with no such choice, male and female tissues were consumed to similar degrees (one-way ANOVA: $F_{1,47} = 2.27$, SS = 24 497, $P = 0.139$, Fig. 3c). Phlorotannins were more abundant in male tissues used in these summer trials (male $\bar{x} = 3.96\%$ DW, S.E. = 0.13; female $\bar{x} = 3.35\%$ DW, S.E. = 0.27; Paired *t*-test, $T_7 = 3.31$, $P < 0.05$). Conversely, during the winter no preference for either sex was observed (one-way ANOVA: $F_{3,92} = 0.78$, SS = 15 828, $P = 0.507$, Fig. 3d), and phlorotannin levels between the two sexes were similar (male $\bar{x} = 3.93\%$ DW, S.E. = 0.20; female $\bar{x} = 4.26\%$ DW, S.E. = 0.36; Paired *t*-test, $T_7 = 0.78$, $P = 0.460$) in the *A. nodosum* tissues presented to the grazers.

During summer feeding trials, Bull *L. obtusata* consumed female tissue preferentially to male tissue irrespective of treatment (one-way ANOVA: $F_{3,92} = 2.93$, SS = 204 300, $P < 0.05$, Fig. 3e), and male tissues ($\bar{x} = 4.00\%$ DW phlorotannin, S.E. = 0.23) used in these trials were more heavily defended than female tissues ($\bar{x} = 3.41\%$ DW phlorotannin, S.E. = 0.2; Paired *t*-test, $T_7 = 2.40$, $P < 0.05$). Consistent with both Menai and Cemlyn trials, no preference for either sex was observed during the winter (one-way ANOVA: $F_{3,92} = 1.7$, SS = 34 577, $p = 0.172$, Fig. 3f),

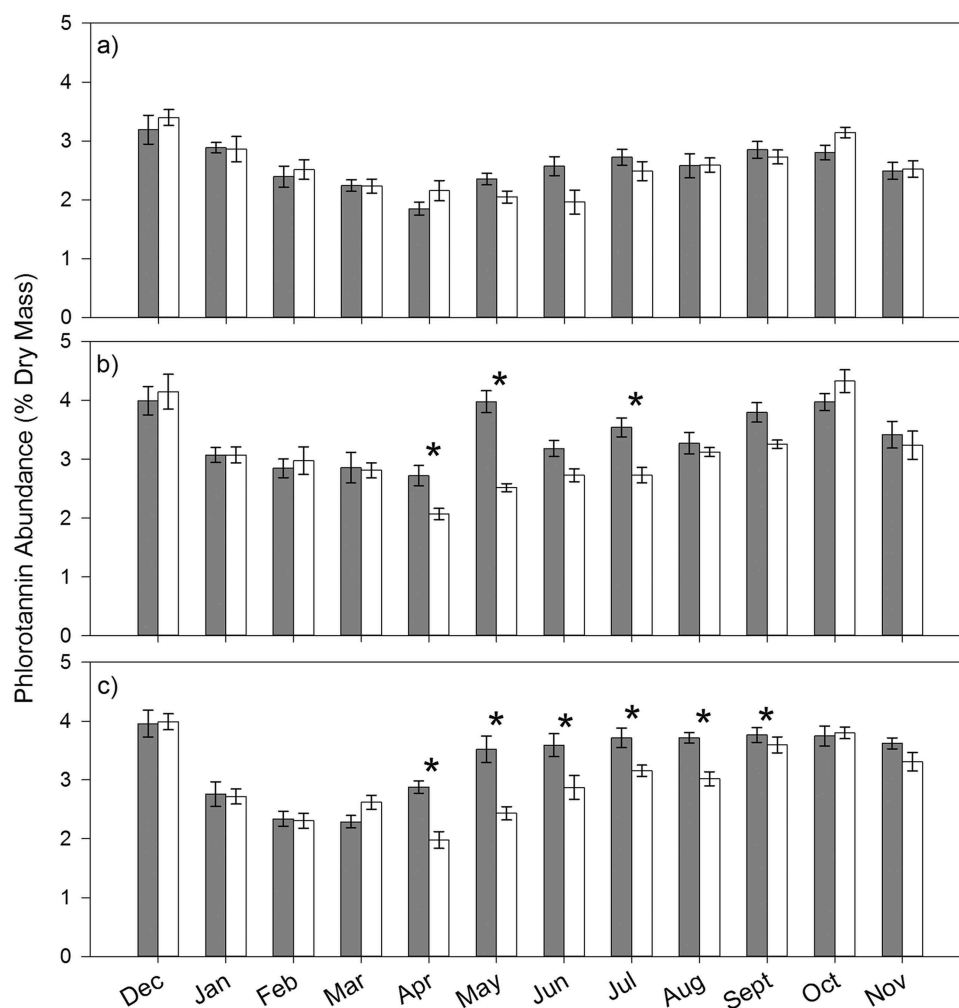


Fig. 2. Phlorotannin abundance (% dry mass) in the meristematic tissues of male (grey bars) and female (white bars) *A. nodosum* collected from (a) Menai, (b) Cemlyn and (c) Bull across a 12-month period from December to November. Each bar represents 10 individuals. Error bars show ± 1 S.E. * indicates that differences between males and females were significant based on Tukey's HSD *post hoc* tests ($P < 0.05$).

and the phlorotannin abundances within tissues from both sexes were similar during this time (male $\bar{x} = 3.67\%$ DW, S.E. = 0.17; female $\bar{x} = 3.80\%$ DW, S.E. = 0.12; Paired *t*-test, $T_7 = 0.94$, $P = 0.377$).

Plasticity in resource allocation

In general, growth variables and RRI exhibited more plasticity than others such as energy in receptacles and defence allocation (Fig. 4). There was a significant difference between males and females in their allocation of energy to the receptacles (one-way ANOVA: $F_{1,4} = 49$, $SS = 0.00082$, $P < 0.01$, Fig. 4), although the levels of estimated plasticity exhibited were low (male $\bar{x} = 5\%$, female $\bar{x} = 7.3\%$). For all other variables, no significant differences in RDPI were detected between males and females.

Discussion

This study demonstrates that sex-specific reproductive trade-offs can occur in *A. nodosum*, although they are

variable with time and manifest differently between sites. Males and females consistently differed in their reproductive investments, with females generally investing more energy and/or defences into receptacles than males. Female plants often invest more into reproduction (Hesse & Pannell, 2011a) and this is also probably typical for other brown algae (Vernet & Harper, 1980). Both sexes reduced vegetative defensive investment during the reproductive period and increased production over the summer, in accordance with previous annual surveys of phlorotannin production (e.g. Ragan & Jensen, 1978). This was probably to compensate for increased UV exposure (Pavia *et al.*, 1999) and elevated herbivory that can occur during summer (e.g. Newell *et al.*, 1971). Whilst males were able to increase their defensive allocation immediately after gamete release, females were unable to produce the same amount of phlorotannins as males for 3–6 months in two of the populations sampled. Annual patterns of resource allocation are known to display by-sex differences in many plants, owing to the differential costs of reproduction (e.g. Ågren, 1988; Cipollini

Table 3. Phlorotannin concentration (% of dry mass) of meristematic tissues from *A. nodosum* males and females collected from (a) Menai, (b) Cemlyn and (c) Bull, across a 6-month period from April to September (summer) analysed using two-way ANOVA. * indicates results from Tukey's HSD *post hoc* analyses for significant effects, largest reported first (groups that do not share a number are significantly different).

(a)				
	DF	MS	F	P
Sex	1	0.76	3.46	= 0.066
Month*	5	1.77	8.11	< 0.05
Sex × Month	5	0.48	2.19	= 0.061
Residual	108	0.22		

*Month: (1) Sep; (1, 2) Jul, Aug; (2, 3) Jun, May; (3) Apr.

(b)				
	DF	MS	F	P
Sex	1	0.76	75.16	< 0.05
Month	5	2.91	15.84	< 0.05
Sex × Month*	5	0.98	5.36	< 0.05
Residual	108	0.18		

*Sex × Month. (1) M May; (1, 2) M Sep; (1, 2, 3) M Jul; (2, 3, 4) M Aug, F Sep, M Jun; (3, 4, 5) F Aug; (4, 5) F Jul, F Jun, M Apr; (5, 6) F May; (6) F Apr.

(c)				
	DF	MS	F	P
Sex*	1	14.26	63.64	< 0.05
Month**	5	3.83	17.10	< 0.05
Sex × Month	5	0.49	2.19	= 0.061
Residual	108	0.22		

*Sex: (1) M; (2) F.

**Month: (1) Sep; (1, 2) Jul; (1, 2, 3) Aug; (2, 3) Jun; (3) May; (4) Apr.

Table 4. Phlorotannin concentration (% of dry mass) of meristematic tissues from *A. nodosum* males and females collected from (a) Menai, (b) Cemlyn and (c) Bull, from December to March and October to November (winter) analysed using two-way ANOVA. * indicates results from Tukey's HSD *post hoc* analyses for significant effects, largest reported first (groups that do not share a number are significantly different).

(a)				
	DF	MS	F	P
Sex	1	0.37	1.57	= 0.212
Month*	5	3.06	13.11	< 0.05
Sex × Month	5	0.10	0.43	= 0.825
Residual	108	0.23		

*Month: (1) Dec, Oct; (1, 2) Jan; (2, 3) Nov, Feb; (3) Mar.

(b)				
	DF	MS	F	P
Sex	1	0.15	0.35	= 0.558
Month*	5	6.74	15.85	< 0.05
Sex × Month	5	0.17	0.40	= 0.847
Residual	108	0.23		

*Month: (1) Oct, Dec; (2) Nov, Jan, Feb, Mar.

(c)				
	DF	MS	F	P
Sex	1	< 0.01	< 0.01	= 0.954
Month*	5	50.09	46.05	< 0.05
Sex × Month	5	0.21	0.98	= 0.435
Residual	108	0.22		

*Month: (1) Dec; (1, 2) Oct; (2) Nov; (3) Jan, Mar, Feb.

Table 5. Investment into receptacle defence (phlorotannin abundance in receptacles) normalized for investment into vegetative defence (phlorotannin abundance in meristems) for *A. nodosum* from (a) Menai, (b) Cemlyn and (c) Bull, collected over a 5-month period from December to April. * indicates results from Tukey's HSD *post hoc* analyses for significant effects, reported largest first (groups that do not share a number are significantly different).

(a)				
	DF	MS	F	P
Sex	1	0.04	1.88	= 0.173
Month*	4	0.09	3.87	< 0.05
Sex × Month	4	0.02	0.93	= 0.452
Residual	90	0.02		

*Month: (1) Apr, Mar; (1, 2) Feb, Dec; (2) Jan

(b)				
	DF	MS	F	P
Sex	1	0.16	7.73	< 0.05
Month	4	0.09	16.80	< 0.05
Sex × Month*	4	0.09	4.68	< 0.05
Residual	90	0.02		

*Sex × Month: (1) F Apr; (1, 2) F Feb; (1, 2, 3) M Feb, F Jan; (2, 3) M Mar; (2, 3, 4) F Mar; (3, 4, 5) M Apr, M Jan; (4, 5) M Dec; (5) F Dec.

(c)				
	DF	MS	F	P
Sex	1	0.09	5.34	< 0.05
Month	4	0.08	4.35	< 0.05
Sex × Month*	4	0.05	2.86	< 0.05
Residual	90	0.02		

*Sex × Month: (1) F Apr; (1, 2) F Feb; (1, 2, 3) M Feb, M Jan, F Mar, F Jan, M Mar; (2, 3) F Dec, M Apr; (3) M Dec.

et al., 2013). These results are in accordance with the resource allocation hypothesis, which states that an increase in resource expenditure in growth, defence or reproduction must lead to a decrease in resource expenditure in one or more of the other allocations (Coley *et al.*, 1985; Boecklen & Hoffman, 1993; Cornelissen & Stiling, 2005).

In accordance with previous studies, significant differences in phlorotannin abundance led to greater consumption by *Littorina obtusata* in both choice (e.g. Pavia *et al.*, 2002) and no-choice (e.g. Toth *et al.*, 2007) trials at Bull during the summer. However, at Cemlyn females were consumed more in choice trials only. Densities of snails were higher in the choice trials and this may have influenced feeding

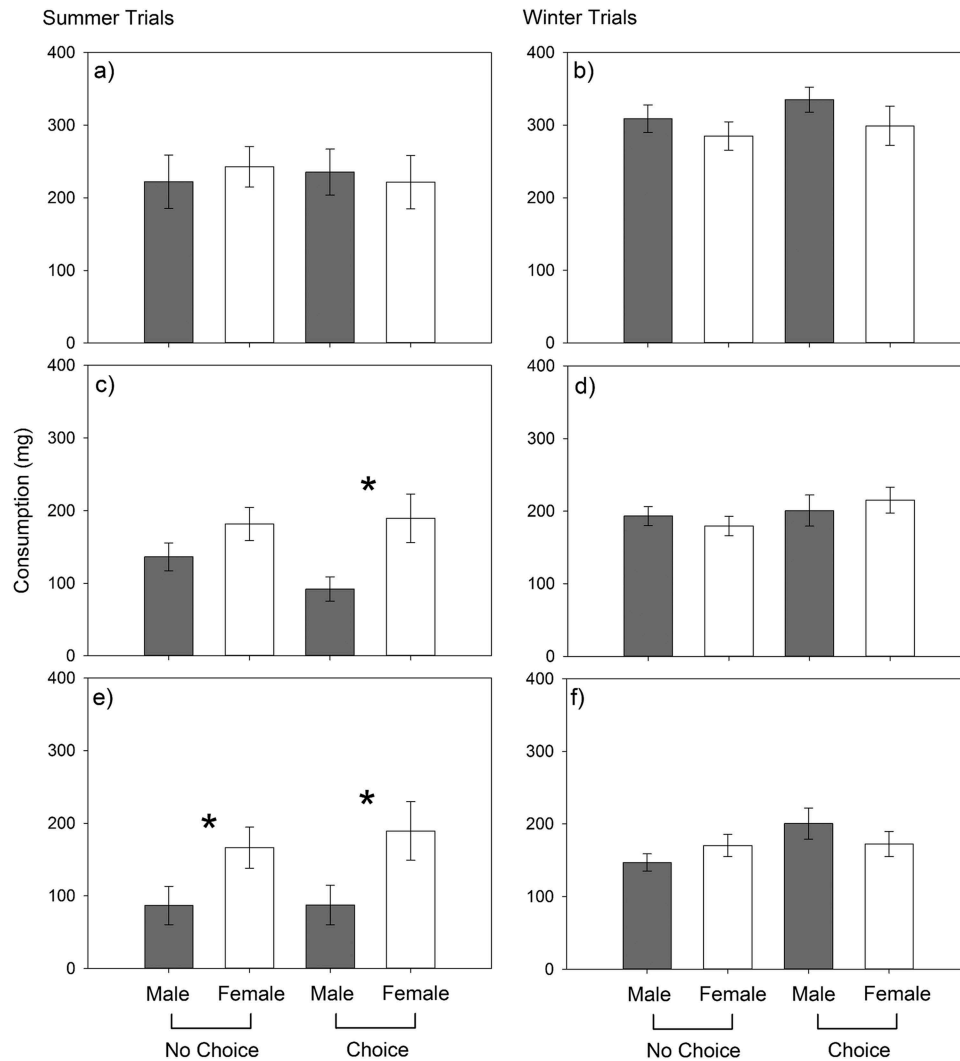


Fig. 3. Consumption (change in algal mass corrected for autogenic changes in mass) of male (grey bars) and female (white bars) meristematic *A. nodosum* tissues from Menai in summer (a), Menai in winter (b), Cemlyn in summer (c), Cemlyn in winter (d), Bull in summer (e), Bull in winter (f), by *Littorina obtusata* (3 grazers per 500 mg \pm 50 mg) in 10-day feeding trials. Each bar represents 24 replicates from eight algal individuals ($n = 192$), error bars show ± 1 S.E. * indicates when differences in grazing rate were significant, based on one-way ANOVA (see text for statistics).

behaviour. However, it is unclear why the Cemlyn and Bull trials differed in this regard and these results should be interpreted with caution. Despite the apparent vulnerability of females to herbivory, there were no differences in size or abundance between the sexes in the field. Growing large is of paramount importance to the competitive and reproductive ability of *A. nodosum* (Cousens, 1985) and therefore it is unsurprising that females do not appear to sacrifice investments into growth.

Although it is clear that females should be the same size as males to avoid a competitive disadvantage, it is far less clear how they are able to achieve this given they may be more susceptible to herbivory for around half of the year. In plants, females employ a number of physiological and ecological strategies to compensate for their increased expenditure in reproduction, including higher capacities for photosynthesis and nutrient uptake (Hesse & Pannell, 2011a; Delph & Herlihy, 2012). Some populations of plant

also display sex-specific microhabitat partitioning, where individuals only grow in specific patches of a particular habitat suitable for their requirements (Sánchez-Vilas & Pannell, 2010). Beneficial inter-specific and intra-specific microhabitat partitioning, other than that caused by intertidal zonation, is known to occur in seaweeds (Taylor & Hay, 1984; Stachowicz *et al.*, 2008). It is conceivable that microhabitat partitioning by females could allow them to compensate for their defensive handicap in the field, since densities of gastropod herbivores can vary at very small spatial scales (< 1 m, Forrest *et al.*, 2001).

Relative distance plasticity indices (RDPIs) were comparable to those calculated for higher plant traits, also compared over a small number (2–3) of populations (e.g. Warren & Lake, 2013). The RDPIs differed between males and females in their allocation of energy to reproductive tissues, but not when considered relative to their respective dry-weights (relative reproductive investments, RRI). This suggests that

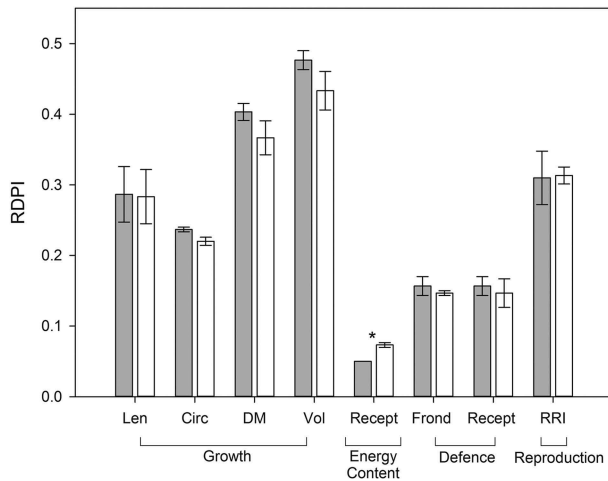


Fig. 4. Relative Distance Plasticity Index (RDPI) between sites for responses in growth (Len = length, Circ = circumference, DM = dry mass, Vol = volume (length \times circumference²), ash-free dry mass in receptacles, defence in terms of phlorotannin production in fronds and receptacles, and reproductive effort (Relative Reproductive Index) for male (grey bars) and female (white bars) *A. nodosum*. Error bars show ± 1 S.E. * indicates when differences in RDPI between males and females were significant, based on one-way ANOVA (see text for statistic).

although between-site variability in the cost of individual receptacles is determined slightly more through plasticity in females than males (albeit with very small values), ultimately the total investment into reproduction is equally constrained by genetics in each sex. Therefore, when sex-specific trade-offs do manifest, they are probably because of physiological constraints brought about through the costs of reproduction at any given site, and not because of flexibility or ‘choice’ on the part of any one individual. Interestingly, at the two sites where sex-specific reproductive trade-offs manifested, females did not reduce phlorotannin production in their reproductive structures in parallel with production in the vegetative tissues over spring. This resulted in the female’s receptacles being relatively better defended than those of males immediately before gamete release. Pavia *et al.* (2002) report that *A. nodosum* concentrate defences in tissues with the greatest ‘fitness value’ (i.e. the tissues with the greatest contribution to success), and this suggests that female receptacles are more valuable than those of males.

Previous studies in terrestrial environments have shown that differences between the sexes increase in magnitude at more stressful habitats, both in purely dioecious populations and those exhibiting subdioecy (e.g. Elmqvist & Gardfjell, 1988; Boecklen & Hoffman, 1993; Dawson & Ehleringer, 1993; Kohorn, 1995). In plants this often manifests in disparities in size or morphology, although this is not always the case (e.g. Sakai & Sharik, 1988; Delph, 1990). It is notable that at the site with least wave and herbivore pressure, sex-specific

reproductive trade-offs in vegetative defence were absent, and that they were more pronounced at the site with greatest wave-action and herbivore abundances. Drawing conclusions about the influence of stress on sex-specific reproductive trade-offs is not possible from this study because the stress conditions were not replicated, although this would be an interesting avenue for future investigation. In *A. nodosum*, low germling survival rates are associated with increased biotic and abiotic stress (Vadas *et al.*, 1990; Cervin & Åberg, 1997; Dudgeon *et al.*, 2001). Although the ratio of antheridia (which release sperm) to eggs is not known for *A. nodosum*, in three closely related species (*Fucus vesiculosus*, *F. spiralis* and *F. serratus*) ratios are known to be between 40 and 400, to 1 (Vernet & Harper, 1980). It is possible that germling mortality driven by wave action and/or herbivory at ‘high stress’ sites results in the selection of females that prioritize reproduction over defence. If eggs are indeed limited compared with sperm in this species, it would explain why a similar selection pressure is not experienced by males.

Growing in dense, gregarious stands is vital to the success of *A. nodosum*, and positively benefits survival of germlings (Dudgeon & Petraitis, 2005). Being dioecious should facilitate a gregarious life-history (e.g. Dorken & Barrett, 2003; Eppeley & Pannell, 2007), but adaptations are required to overcome reproductive handicaps. Research into photosynthetic ability, capacity for nutrient uptake, microhabitat partitioning, and the influence of stress on reproductive trade-offs is needed to fully understand the wider ecological impacts of sex-specific reproductive trade-offs in macroalgae (Hesse & Pannell, 2011a; Delph & Herlihy, 2012).

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Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article’s online page at <https://doi.org/10.1080/09670262.2017.1328746>

Supplementary fig. 1. Modelled wave fetch distances for a) Cemlyn Bay (solid lines) and Bull Bay (dashed lines) and b) Menai Bridge (inset), showing the limited fetch as solid lines. Wave stress metrics for Menai, Cemlyn, and Bull sites, quantified as c) mean fetch distances, and d)

percentage change of Plaster of Paris clods left *in-situ* for 12 tidal cycles in August.

Supplementary fig. 2. Site stress metrics quantified as a) Sediment grain-size analysis, and b) Estimated abundance of known herbivores of *Ascophyllum nodosum*; Ll = *Littorina littorea*, Lo = *Littorina obtusata*, Lf = *Littorina fabalis*, Psp = *Patella* sp.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

M. Kurr: original concept, sample collection, chemical analysis, feeding trials, drafting and editing manuscript; A. Davies: original concept, calculation of Relative Distance Plasticity Indices, editing manuscript.

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